

# **Preventative topical diclofenac treatment differentially decreases tumor burden in male and female Skh-1 mice in a model of UVB-induced cutaneous squamous cell carcinoma**

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## **Introduction**

According to recent SEER Cancer Statistics, between 2005-2009 the overall cancer incidence and mortality rate was higher in men than in women [1]. This sex difference is also observed in non-melanoma skin cancer (NMSC), which includes both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Epidemiological studies demonstrate that men have twice the incidence of BCC and three times the incidence of SCC as women [2]. There are over 3.5 million NMSC diagnoses in approximately 2 million patients each year [3] with treatment costs reaching over \$1.4 billion [4], making it the fifth most costly cancer in the Medicare population [5]. While SCC only makes up 16% of all NMSC, it can metastasize more frequently, killing approximately 2500 people each year, of which men are the majority [6].

A component of solar radiation, UVB is the major environmental carcinogen contributing to the development of both BCC and SCC [7-9]. Though the increased SCC incidence observed in men has been attributed to lifestyle differences with higher levels of sun exposure and less protection from the sun compared to women [10-12], we have previously shown using a murine model that when exposed to equivalent UVB doses, this sex bias still holds true [13]. With the increasing incidence of NMSC and the knowledge that men and women respond differently to UVB, it is imperative to examine the efficacy of potential treatments in both sexes.

It is well documented that UVB exposure is associated with an inflammatory response [14-16], which following both acute and chronic exposure, is known to play a role in the

formation of NMSC [17-19]. Chronic UVB exposure induces constitutive expression of cyclooxygenase 2 (COX-2), which is the primary source of elevated prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the skin [20,21]. This prostaglandin synthesis increase plays a key role in carcinogenesis by contributing to uncontrolled proliferation of damaged cells that ultimately form tumors [22-26]. The inflammatory response that coincides with elevated PGE<sub>2</sub> levels is linked to increased DNA damage, which following exposure to stimuli such as UVB, induces the expression of wild type p53, a tumor suppressor gene [27]. The loss of function of p53 by mutation or allele loss is a common finding in many human malignancies, including cutaneous SCC [28,29].

UVB exposure induces the production of reactive oxygen species such as hydrogen peroxide, which if not maintained at a homeostatic level by antioxidant networks, can cause oxidative stress and cellular damage. The main cutaneous antioxidant, catalase, has the role of detoxifying hydrogen peroxide. Skin antioxidant activity decreases with UVB exposure, and specifically, catalase activity level decreases have been associated with both skin carcinogenesis and progression [30-36]. Previously, we reported decreased antioxidant activity [13], and specifically, catalase activity [37], in the skin of male mice compared to female mice that may contribute to increased tumorigenesis in males.

We previously demonstrated that treating female mice topically with the COX-2 inhibitor, celecoxib, decreased PGE<sub>2</sub> levels as well as the percentage of p53-positive epidermal cells in the acute UVB-induced cutaneous response [38]. Chronic UVB exposure and subsequent topical celecoxib treatment decreased both tumor number and grade in female mice; however, its efficacy in males has not been described. Since male mice display lower levels of inflammation and antioxidant activity as compared to female mice after UVB exposure [13,37], we sought to investigate the extent to which a topical anti-inflammatory drug would

affect tumor burden as well as PGE<sub>2</sub> or catalase levels in the skin of male versus female mice after chronic UVB exposure.

Because celecoxib is not available to patients in a topical formulation, we conducted studies using topical diclofenac, an NSAID and COX-2 inhibitor, which is currently prescribed to patients with actinic keratotic lesions—precursors of SCC. Topical formulations are better tolerated and cause fewer adverse effects compared to oral COX inhibitors as the systemic levels are greatly decreased with topical treatments [39]. Open label studies have demonstrated that diclofenac is indeed well tolerated and effective short-term for treating actinic keratotic lesions [40,41]. However, its efficacy as a preventative agent in patients without evidence of precursor lesions but with significant UVB-induced cutaneous damage has not been examined. Additionally, potential differences between men and women in the efficacy of diclofenac treatment have not been reported.

A recent case-control study suggested a dose-dependent, cumulative, protective effect for the reduction of skin cancer risk, specifically SCC, with the use of a number of NSAIDs [42]. In the current study, we have modeled both male and female compliant patients who eliminated sun exposure and began using topical diclofenac regularly to reverse existing damage resulting from chronic UVB exposure and prevent tumor development. Our results demonstrate that despite the observed sex differences in the inflammatory response, prolonged topical diclofenac treatment of chronically UVB-damaged skin effectively reduced tumor multiplicity in male and female Skh-1 hairless mice. Interestingly, with topical diclofenac treatment, tumor burden was significantly decreased and tumors were of lower histologic grade only in male mice. While PGE<sub>2</sub> levels were decreased in both sexes, levels of the antioxidant, catalase, were increased with diclofenac treatment in male mice only. These data support inflammation as a key factor contributing to UVB-induced tumor development in both male and female mice. Taken together, our study emphasizes differences

in the response of male and female murine skin and demonstrates a potential new therapeutic use for this currently available topical treatment as a preventative intervention for patients predisposed to cutaneous SCC development.

## **Materials and methods**

### *Mice*

Outbred, male and female Skh-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee before the initiation of any studies. Mice were dorsally exposed to 2240 J/m<sup>2</sup> UVB, previously determined to be 1 MED, 3× weekly on non-consecutive days for 10 weeks. UVB dose was calculated using UVX radiometer and UVB sensor (UVP, Upland, CA) and emitted by Phillips FS40 UV bulbs (American Ultraviolet Company, Lebanon, IN). Mice were treated topically with vehicle (Surgilube®; Savage Laboratories, Melville, NY) or 500 µg diclofenac (Solaraze®, 3% diclofenac sodium; Doak Dermatologics, Fairfield, NJ) in vehicle for 15 weeks with no additional UVB exposure. This dose of diclofenac was chosen based on previous studies in our laboratory. Surgilube is an inert vehicle used clinically as a surgical lubricant. Tumors larger than 1 mm in diameter were measured weekly with calipers. After sacrifice, 0.5cm<sup>2</sup> section of dorsal skin and all tumors were fixed as previously described [37] while remaining dorsal skin was snap frozen in liquid nitrogen.

### *Tumor Grading*

H&E stained tissue sections of tumors isolated from male and female mice were graded in a blinded manner by a board-certified veterinary pathologist (DFK) as previously described [13].

### *Catalase Activity Assay*

Frozen dorsal skin was crushed and 15 mg was used for analysis of catalase activity using the Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions.

### *Prostaglandin E<sub>2</sub> EIA*

Frozen dorsal skin was crushed and 15 mg was used for analysis of PGE<sub>2</sub> levels using the Prostaglandin E<sub>2</sub> EIA Kit-Monoclonal (Cayman Chemical) according to manufacturer's instructions.

### *Immunohistochemistry*

p53: Paraformaldehyde-fixed/OCT-embedded dorsal skin sections were cut (10 µm) onto Superfrost Plus® microscope slides (Fisher Scientific) and stored at -80°C for future analysis. Slides were thawed overnight at room temperature, baked at 60°C for 30 min, and then rehydrated in Clear Rite 3 (Richard-Allen Scientific) and a graded series of ethanol. Sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 25 min in a vegetable steamer reaching 95°C. Slides were cooled for 20 minutes at room temperature and blocked in 1× Casein (Vector Laboratories) for 10 minutes followed by incubation with primary p53 antibody (clone CM5p, Novocastra (Leica Microsystems Inc.), Buffalo Grove, IL) at a 1:500

dilution in 1× Casein at room temperature for 1 hour. Slides were incubated in Rabbit Link and Label (Biogenex, Fremont, CA), each for 30 minutes at room temperature. Slides were washed in PBS containing 0.05% Tween (PBST) between the various incubation steps described above. Following a final PBST wash, slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at room temperature. Slides were washed in deionized water, counterstained with Hematoxylin 2, and dehydrated in an increasing series of ethanol followed by Clear Rite 3, and then cover slipped with VectaMount mounting medium (Vector Laboratories). p53 foci were counted as 3 or more adjacent p53-positive cells and examined in 5 fields of view at 20× magnification.

Ki67: Protocol is the same as above through incubation with 1× Casein. Slides were incubated with primary Ki67 antibody (Dako, Carpinteria, CA) at a 1:200 dilution in 1× Casein overnight at 4°C in a humid chamber. Slides were incubated in biotinylated IgG (Vector Laboratories) at a 1:200 dilution in 1× Casein for 30 minutes at room temperature. Slides were incubated in ABC Elite (Vector Laboratories) for 30 minutes at room temperature. Slides were washed in PBST between the various incubation steps described above. Slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at RT. Slides were washed in deionized water, counterstained, and dehydrated as above. Ki67-positive cells were examined in 5 fields of view at 60× magnification.

### *Statistical Analysis*

The results presented in this paper were part of a larger experiment involving four treatment groups (of which Diclofenac was one) and a single control group. Dunnett's adjustment [43,44] for multiplicity was used for comparing the primary outcome of tumor burden at 24 weeks between the treatment groups and control in order to restrict the probability of a type I error to 5%. The number of control mice was inflated compared to the treatment groups to

increase the power of the comparison [43]. Residual plots verified the model assumptions of normality and homoscedasticity and a logarithmic transformation was utilized if necessary. Continuous outcome data were analyzed using an ANOVA approach with linear contrasts for testing the comparisons of interest. A mixed-effects regression model with a random slope and intercept by subject was used to model tumor growth from the time of tumor origination. For count data, Poisson regression was used. All analyses were conducted in SAS version 9.2 (SAS Institute, Cary, NC).  $p$ -values  $\leq 0.05$  were considered statistically significant.

## Results

### *Diclofenac topical treatment decreased tumor number and burden*

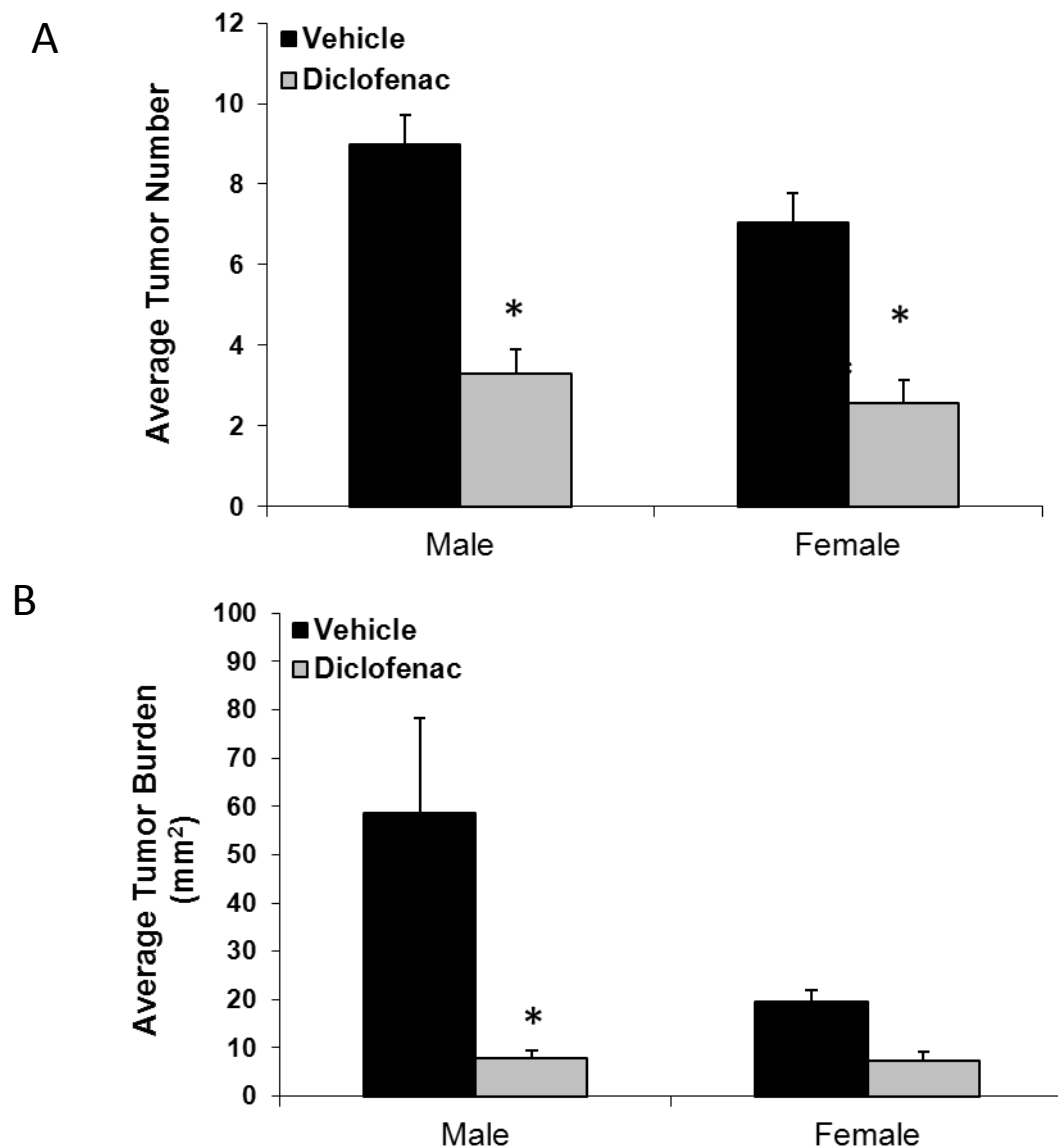
To examine the effects of topical diclofenac treatment as a preventative agent against tumor development, we exposed male and female Skh-1 hairless mice to 2240 J/m<sup>2</sup> UVB (previously determined to be 1 MED in our laboratory) for ten weeks to model chronic sun exposure. Mice were then topically treated with either surgilube (vehicle) or diclofenac for 15 weeks without further UVB exposure to model a lifestyle change. Non-irradiated male and female mice treated with either vehicle or diclofenac did not develop tumors. Additionally, mice that received no vehicle treatment did not exhibit a significantly different tumor burden compared to mice treated with vehicle, indicating that the vehicle had no significant effect on tumorigenesis in this study (data not shown).

Following 10 weeks of only UV exposure and 15 weeks of preventative topical treatment with diclofenac, male mice exhibited decreased tumor multiplicity with 57.8% fewer tumors compared to the mice treated with vehicle ( $p < 0.0001$ , Figure 1A). At week 24, male mice treated topically with diclofenac exhibited significantly decreased tumor burden by 82.5% ( $p < 0.0001$ ) compared to the mice treated with vehicle (Figure 1B). Female mice treated topically with diclofenac also demonstrated decreased tumor multiplicity with 63.1% times fewer tumors compared to mice treated with vehicle ( $p < 0.0001$ , Figure 1A). Female mice

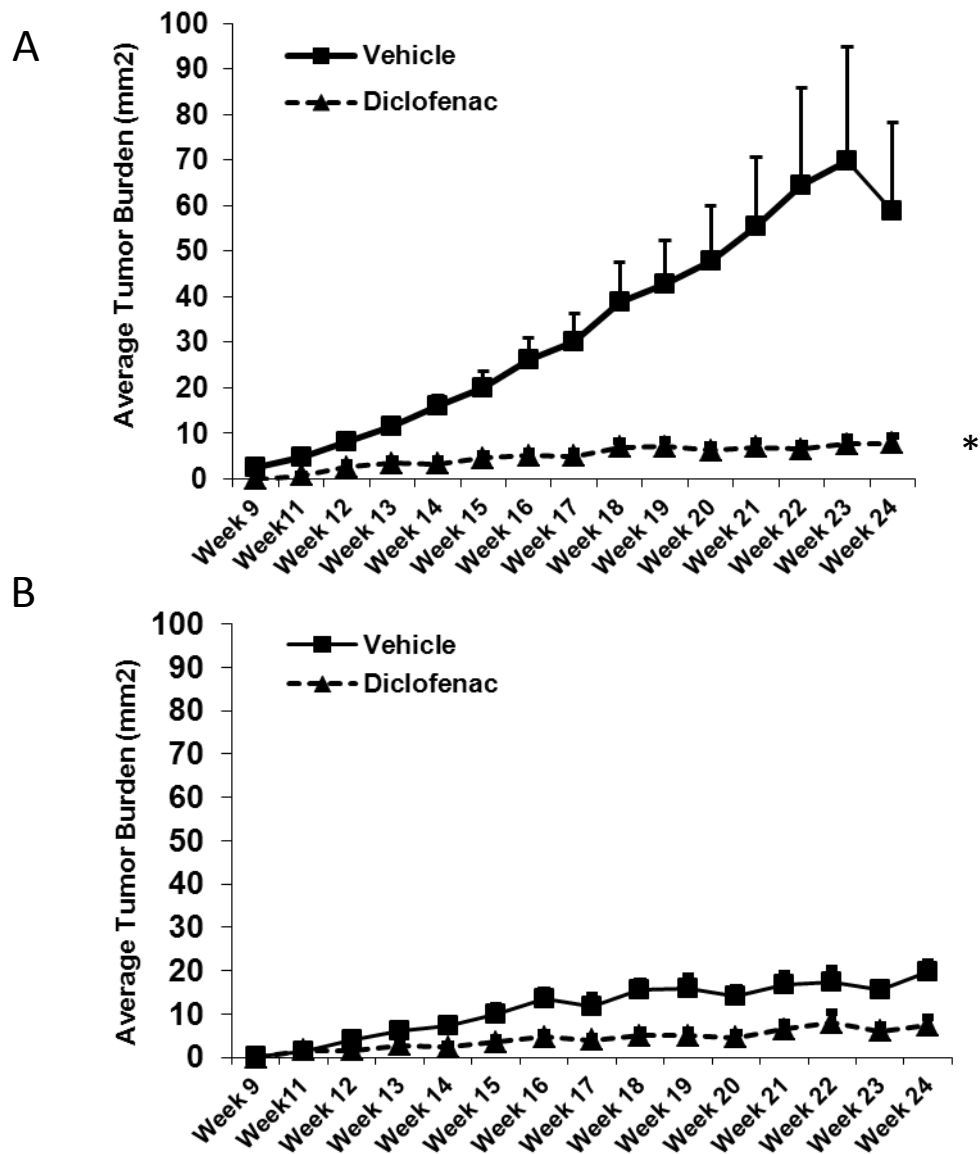
treated topically with diclofenac displayed a 51.7% decrease in average tumor burden that was not statistically different ( $p=0.1098$ ) compared to mice treated with vehicle (Figure 1B). However, female mice treated with vehicle exhibited approximately half the tumor burden as vehicle-treated males, which was significantly different ( $p=0.0033$ ).

Examining the change in tumor burden over time, we found that male mice treated topically with diclofenac had a significantly decreased tumor growth rate of 6.9% while mice treated with vehicle exhibited a growth rate of 15.9% ( $p=0.0057$ , Figure 2A). Though female mice treated topically with diclofenac also exhibited a slight decrease in tumor growth rate compared to mice treated with vehicle, 8.6% versus 11.1%, respectively, this decrease was not significant ( $p=0.3949$ , Figure 2B).





**Figure 1 Diclofenac decreases tumor multiplicity and burden in male and female mice.** Tumors with a diameter larger than 1 mm were measured weekly. (A) Male and female mice treated topically with diclofenac for 15 weeks after 10 weeks of UVB exposure exhibited decreased tumor multiplicity compared to the mice treated with vehicle (\* $p < 0.0001$ ). (B) At week 24, male mice treated topically with diclofenac exhibited significantly decreased tumor burden (\* $p < 0.0001$ ) compared to the mice treated with vehicle while the decrease in female mice was not statistically significant ( $p = 0.1098$ ).



**Figure 2 Diclofenac decreases tumor growth rate in male mice.** Tumors with a diameter larger than 1 mm were measured weekly. (A) Male mice treated topically with diclofenac had a decreased tumor growth rate ( $p=0.0057$ ) compared to mice treated with vehicle. (B) Female mice treated topically with diclofenac exhibited a slight decrease in tumor growth rate compared to mice treated with vehicle that was not statistically significant ( $p=0.3949$ ).

*Diclofenac topical treatment decreased the severity of tumors in male mice*

Tumors were isolated from mice after 10 weeks of UVB exposure followed by 15 weeks of topical treatment and scored by a board-certified veterinary pathologist (DFK). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. Male mice treated with vehicle developed tumors of each grade while male mice preventatively treated with diclofenac developed tumors graded as papilloma (grades 1-3) or microinvasive SCC (grade 1 only). As seen in Table 1, male mice treated with vehicle, 19.3% of graded tumors were malignant while male mice treated topically with diclofenac developed malignant tumors consisting of only 5.9% of graded tumors. There was a trend towards a lower malignancy rate, but it was not statistically significant ( $p=0.2413$ ). Importantly, male mice treated with diclofenac developed no fully invasive SCC lesions while mice treated with vehicle developed 5.3% of graded tumors that were fully invasive SCC lesions.

	% Premalignant Tumors	% Malignant Tumors
Male Vehicle	80.7	19.3 (5.3% SCC)
Male Diclofenac	94.1	5.9 (No SCC)

Table 1. Distribution of tumor grades in male mice.

Female mice treated with vehicle developed all grades of papilloma, microinvasive SCC grade 1 and fully invasive SCC. Mice preventatively treated with diclofenac developed all grades of papilloma, microinvasive SCC grades 1 and 2, and fully invasive SCC.

Interestingly, while female mice treated topically with diclofenac exhibited decreased tumor burden compared to vehicle-treated mice, the estimated per tumor malignancy rate was 0.25 compared to 0.11 for mice treated with vehicle, which trended towards an increased malignancy rate but was not statistically significant ( $p=0.2574$ ).

	% Premalignant Tumors	% Malignant Tumors
Female Vehicle	89.7	10.3
Female Diclofenac	75.0	25.0

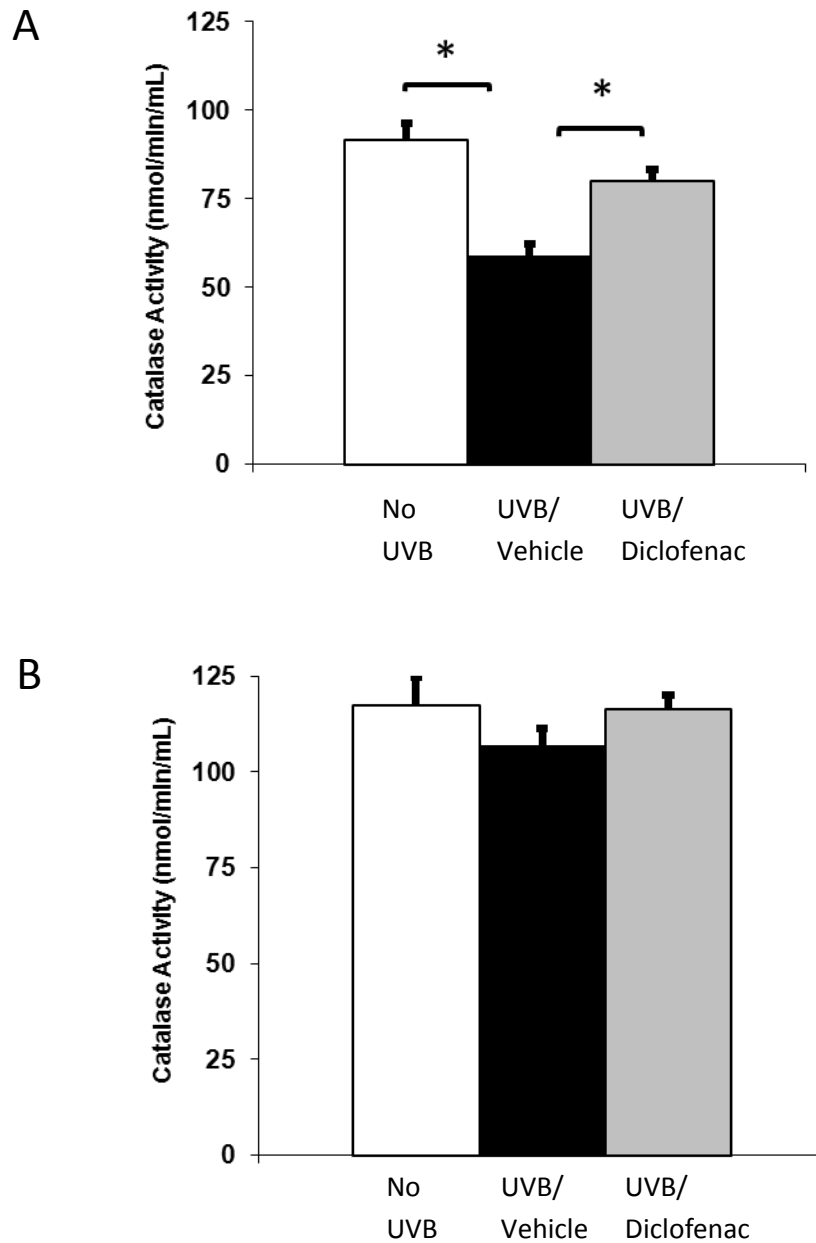
Table 2. Distribution of tumor grades in female mice.

#### *Diclofenac topical treatment increased catalase activity levels in male mice*

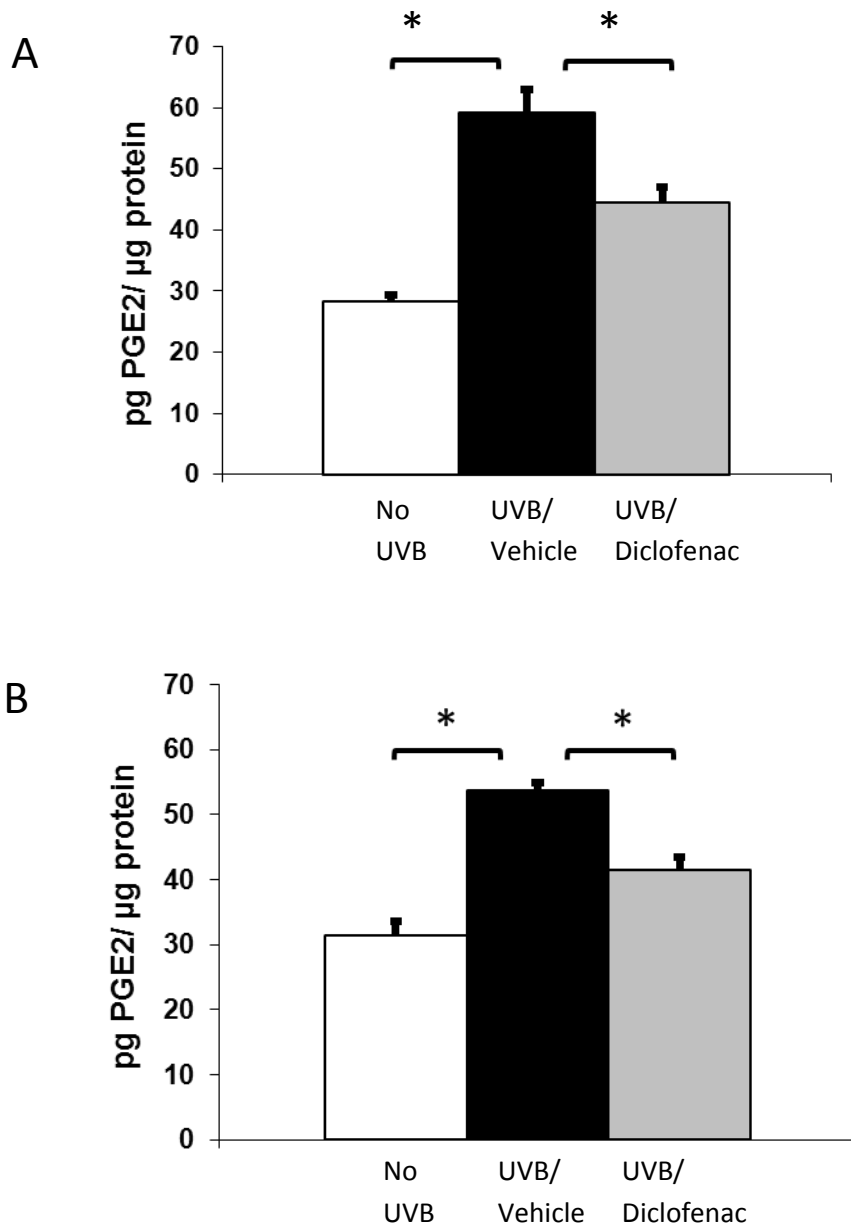
Protein was extracted from tumor-free, dorsal skin in order to examine catalase activity levels. Even after 15 weeks without UVB exposure, catalase activity levels were significantly decreased in the vehicle-treated male skin as compared to the non-irradiated controls ( $p=0.0004$ , Figure 3A), but not in female skin ( $p=0.2121$ , Figure 3B). Topical diclofenac treatment applied to male skin significantly increased catalase activity from levels observed in the vehicle-treated skin ( $p=0.0035$ , Figure 3A), and in fact, catalase activity is almost restored to levels observed in non-irradiated skin. In contrast, catalase activity levels in female skin treated topically with diclofenac were not significantly altered compared to those observed in vehicle-treated skin ( $p=0.1590$ , Figure 3B).

*Diclofenac topical treatment decreased cutaneous Prostaglandin E2 (PGE<sub>2</sub>) levels*

To confirm that topical diclofenac treatment preventatively applied effectively inhibited COX-2-mediated PGE<sub>2</sub> production, cutaneous PGE<sub>2</sub> levels were determined by enzyme immunoassay. In chronically irradiated male (Figure 4A) and female (Figure 4B) vehicle-treated skin, even after 15 weeks without UVB exposure, PGE<sub>2</sub> levels were significantly increased from levels observed in non-irradiated skin ( $p<0.0001$ ). Skin of male (Figure 4A) and female (Figure 4B) mice exposed to UVB for 10 weeks and treated topically with diclofenac for 15 weeks exhibited significantly decreased PGE<sub>2</sub> levels as compared with chronically irradiated, vehicle-treated mice ( $p=0.0001$ ).



**Figure 3 Diclofenac increases catalase activity in male skin.** Even after 15 weeks without UVB exposure, catalase activity levels were significantly decreased in the vehicle-treated male skin (A) as compared to the non-irradiated controls ( $*p=0.0004$ ). With topical diclofenac treatment in male skin, catalase activity levels were significantly increased from levels observed in the vehicle-treated skin ( $*p=0.0035$ ). (B) Catalase activity levels were not significantly altered in female vehicle-treated skin ( $p=0.2121$ ) or diclofenac-treated skin ( $p=0.159$ ).



**Figure 3.5 Diclofenac topical treatment decreases cutaneous PGE<sub>2</sub> levels in male and female mice.** Cutaneous PGE<sub>2</sub> levels were determined by enzyme immunoassay after 10 weeks of UVB exposure and 15 weeks subsequent topical treatment. In both male (A) and female (B) mice, PGE<sub>2</sub> levels were significantly increased with UVB exposure compared to levels observed in unirradiated mice (\* $p < 0.0001$ ). Skin of male (A) and female (B) mice exposed to UVB for 10 weeks and treated topically with diclofenac for 15 weeks exhibited significantly decreased PGE<sub>2</sub> levels as compared with chronically irradiated vehicle-treated skin (\* $p = 0.0001$ ).

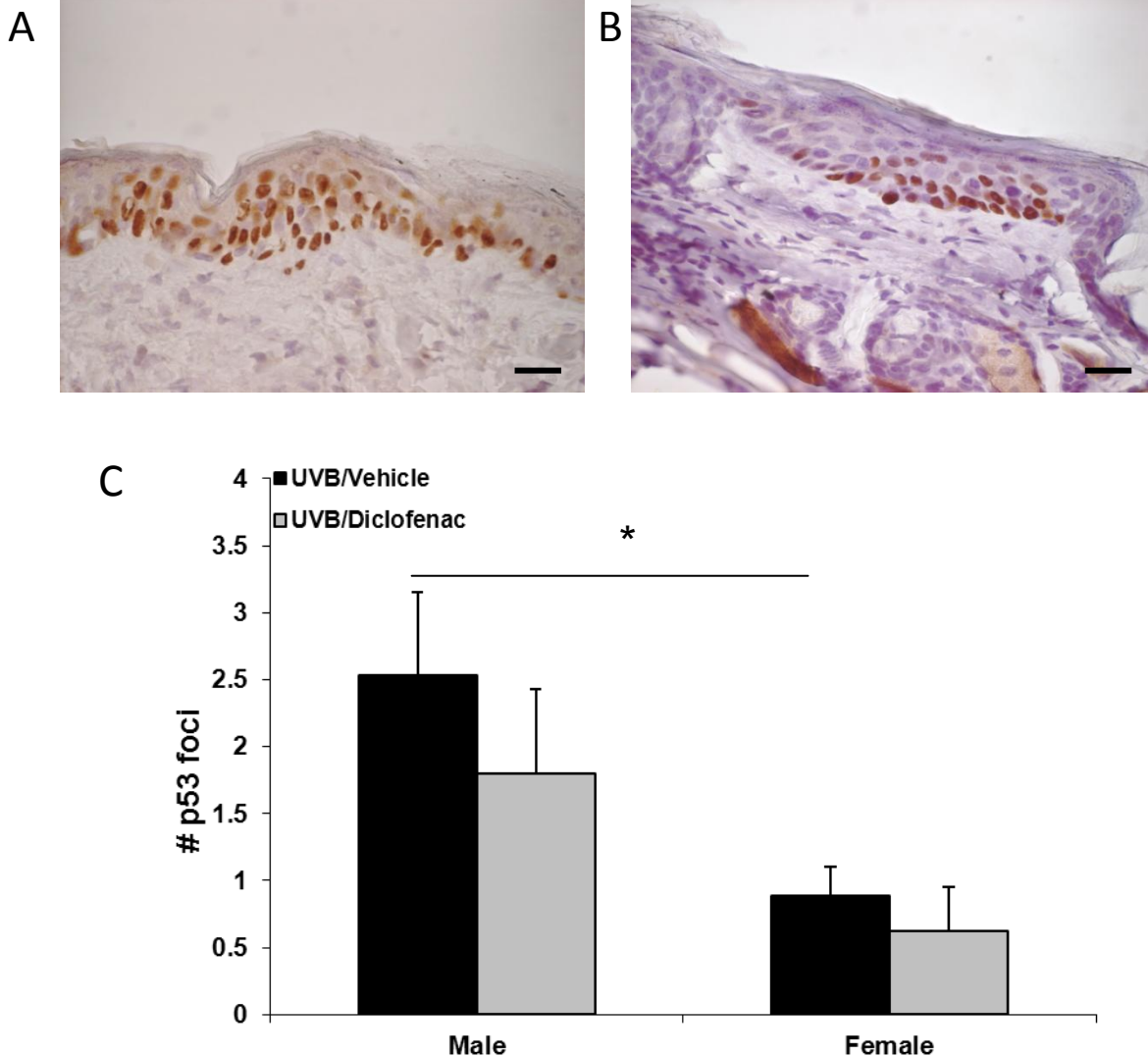
*Male mice have increased numbers of p53-positive foci compared to female mice*

Tumor-free, dorsal skin sections were examined for p53-positive foci via immunohistochemistry with an antibody detecting both wild type and mutant p53 (Figure 5A and B). The number of p53-positive foci measured over five fields was not significantly altered with preventative topical diclofenac treatment in male skin (2.5 for vehicle-treated vs 1.8 for diclofenac-treated,  $p=0.2323$ ) or female skin (0.89 for vehicle-treated vs 0.63 for diclofenac-treated,  $p=0.4918$ ). Corresponding with the increased tumor burden, the mean number of foci was significantly higher in vehicle-treated male skin compared to vehicle-treated female skin (2.5 vs 0.89,  $p=0.0004$ , Figure 5C).

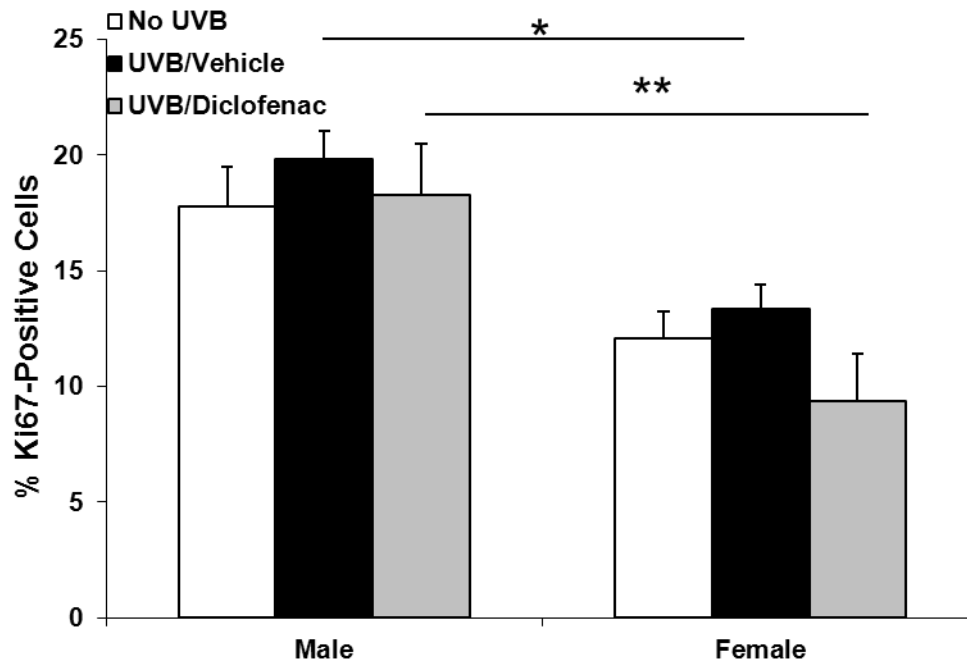
*Diclofenac does not affect proliferation 15 weeks after cessation of UVB exposure*

Tumor-free, dorsal skin sections were stained for Ki67. The percentage of Ki67-positive cells was not different ( $p=0.4708$ ) between the vehicle-treated and diclofenac-treated male skin after 10 weeks of UVB exposure followed by 15 weeks of topical treatment with no additional UVB exposure. In female skin, the percentage of Ki67-positive cells exhibited a decreasing trend from 13.4% to 9.4% with preventative topical diclofenac treatment compared to vehicle-treated mice ( $p=0.0596$ ). Interestingly, the percentage of Ki67-positive cells was significantly higher in male skin compared to female skin with both vehicle and diclofenac topical treatment ( $p<0.006$  and  $p=0.0004$ , respectively, Figure 6).





**Figure 3.6 Male mice have increased numbers of p53-positive foci compared to female mice.** Dorsal, tumor-free, skin sections were examined for p53-positive foci via immunohistochemistry with an antibody detecting both wild type and mutant p53. Representative (A) male and (B) female vehicle-treated skin after 10 weeks of UVB and 15 weeks of treatment. Scale bar = 10 μm. The mean number of foci per field of view was significantly higher (C) in vehicle-treated male skin compared to vehicle-treated female skin (\* $p=0.0004$ ).



**Figure 3.7 Male skin displays higher levels of proliferation regardless of treatment.** Dorsal, tumor-free, skin sections were stained for Ki67. Male skin exhibited higher percentages of Ki67-positive cells compared to female skin with both vehicle (\* $p<0.006$ ) and diclofenac topical treatment (\*\* $p=0.0004$ ).

## Discussion

Our previous studies have demonstrated that when exposed to equivalent, chronic UVB light, male mice exhibit a higher tumor burden compared to female mice [13], which we confirmed in the current study. We also have previously shown that female mice preventatively treated topically with the anti-inflammatory drug, celecoxib exhibited decreased tumor number, grade, PGE<sub>2</sub> levels, and the number of p53-positive cells [20,38]. In the current study, we show that the readily available and currently used NSAID, diclofenac, elicits similar results in male mice treated topically with diclofenac after sustaining significant UVB damage. We demonstrate that prolonged, topical diclofenac treatment of chronically UVB-damaged skin

effectively reduced tumor multiplicity in both sexes. Unexpectedly, tumor burden was only significantly decreased in male mice, where we also observed a slower rate of growth and a trend toward a lower malignancy rate, with no fully invasive squamous cell carcinomas developing in diclofenac-treated male mice. We also observed a sex difference in catalase activity in that male mice treated with diclofenac exhibited increased catalase activity levels compared to vehicle-treated male mice while catalase activity levels in female mice remained unchanged.

The observed increase in catalase activity levels in male skin may be an important factor because we have observed endogenously lower antioxidant activity levels [13] and specifically, lower catalase activity levels in male skin [37]. Additionally, both decreased antioxidant enzyme levels and the resulting oxidative protein damage in chronically UV-exposed skin have been demonstrated [33]. Recently we reported a link between decreased skin catalase activity, increased Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cell infiltration, and increased tumorigenesis in male mice but not in female mice [37]. Elevated levels of PGE<sub>2</sub> have been shown to induce higher levels and more suppressive Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cells that produce high levels of reactive oxygen species (ROS) [45,46]. By decreasing PGE<sub>2</sub> levels in male skin with topical diclofenac treatment, Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cell infiltration is decreased (unpublished data), therefore decreasing myeloid-associated ROS levels. As a result, catalase activity levels may be increased or maintained due to the overall lower levels of cutaneous ROS production. The increased skin catalase activity in male mice treated with diclofenac compared to male mice treated with vehicle may indicate that restoration of this antioxidant is important for decreasing tumorigenesis in male but not in female mice.

In addition to increasing catalase activity, diclofenac topical treatment also reduced the number of p53-positive foci observed in male mice. Though not statistically significant, there is a clear trend towards fewer foci observed. As p53 is indicative of overall DNA damage, a

decrease in the number of p53-positive cells observed demonstrates that diclofenac treatment may be promoting repair or preventing damage altogether. Previous studies demonstrate that ingenol mebutate, a well-tolerated topical antineoplastic drug used for treatment of both NMSC and actinic keratotic lesions [47-49], effectively reduced UVB-induced lesions and p53 patches in Skh-1 mice [50]. However, the side effects of this treatment included scar formation and skin tightening, which were not observed in the current study. Because the mice in the current study were exposed to UVB for 10 weeks and then treated for 15 weeks without further UVB exposures prior to examining tissue, further studies examining various time points in addition to examining levels of mutated p53 would be necessary to determine the role diclofenac is playing in the formation of p53 foci.

Importantly, preventative diclofenac treatment led to decreased tumor burden in male mice and significantly lower PGE<sub>2</sub> levels in both sexes compared to vehicle-treated mice, underscoring the importance of COX-2-mediated inflammation in the UVB-induced carcinogenesis process. PGE<sub>2</sub> also contributes to angiogenesis [51] and invasion [52-55], in addition to inflammation. Notably, angiogenesis has been reportedly increased in actinic keratoses compared to adjacent skin [56]. Further, studies have indicated that an “angiogenic switch” is turned on during this stage, which contributes to the invasive nature and progression of these lesions toward becoming SCCs [57,58]. While not examined in the current study, blocking PGE<sub>2</sub> production with diclofenac treatment may be decreasing angiogenesis and invasive properties that contribute to the lack of development of fully invasive SCC lesions in male mice that are present in the vehicle-treated mice.

We have previously shown that males have lower levels of acute UVB-induced inflammation compared to female mice and suggested that the inflammatory response was not as important to tumorigenesis in male mice [13]. This study offers support to the fact that the inflammatory response in male mice, regardless of magnitude, is indeed important for

tumorigenesis, since decreasing levels of inflammatory mediators and increasing the antioxidant capacity within male skin correlated with decreased tumor number and burden in male mice topically treated with diclofenac.

Recent studies have refocused on the preventative effects of NSAIDs against skin cancer [42,59], highlighting the relevance of our study. While many studies focus on oral NSAIDs that may result in harmful side effects over time, the use of topical NSAIDs results in lower systemic levels and therefore, a lower risk of these side effects including gastrointestinal hemorrhage and peptic ulcer disease [39,60]. Our data demonstrates a potential NSAID alternative for patients with cardiac or other health issues that may be heightened with oral NSAID use.

In summary, we have shown in a model of UVB-induced SCC that topically treating male and female Skh-1 hairless mice prophylactically with the NSAID and COX-2 inhibitor, diclofenac, decreased tumor multiplicity and PGE<sub>2</sub> production in both sexes. In addition, topically applied diclofenac also interestingly increased skin catalase levels and decreased tumor burden as well as the percentage of malignant tumors in male mice. These findings are especially relevant because in humans, men have a greater risk and incidence of cancer overall [1], with a three fold greater incidence of SCC [2]. To the best of our knowledge, this is the first report of a sex-associated difference in the efficacy of topical diclofenac treatment, implying that it may be beneficial to retrospectively dissect out potential sex-based differences in previously reported clinical outcomes in order to more effectively treat each sex. As we previously observed that male mice have lower antioxidant activity as compared with female mice, further studies are needed to determine if the increased catalase levels are contributing to the therapeutic effects of the diclofenac treatment, or if inhibiting inflammation is indeed the primary factor in both sexes. Overall, our data suggest that sustained diclofenac treatment applied topically to chronically UVB-damaged skin before the

appearance of precursor lesions, in combination with the elimination of UVB exposure, may be beneficial to patients who are predisposed to developing SCC.

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